



Ambatipudi, S., Langdon, R., Richmond, R. C., Suderman, M., Koestler, D. C., Kelsey, K. T., Kazmi, N., Penfold, C., Ho, K. M., McArdle, W., Ring, S. M., Pring, M., Waterboer, T., Pawlita, M., Gaunt, T. R., Davey Smith, G., Thomas, S., Ness, A. R., & Relton, C. L. (2018). DNA methylation derived systemic inflammation indices are associated with head and neck cancer development and survival. *Oral Oncology*, 85, 87-94.  
<https://doi.org/10.1016/j.oraloncology.2018.08.021>

Publisher's PDF, also known as Version of record

License (if available):  
CC BY

Link to published version (if available):  
[10.1016/j.oraloncology.2018.08.021](https://doi.org/10.1016/j.oraloncology.2018.08.021)

[Link to publication record in Explore Bristol Research](#)  
PDF-document

This is the final published version of the article (version of record). It first appeared online via Elsevier at <https://www.sciencedirect.com/science/article/pii/S1368837518303117> . Please refer to any applicable terms of use of the publisher.

## University of Bristol - Explore Bristol Research

### General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:  
<http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>



# DNA methylation derived systemic inflammation indices are associated with head and neck cancer development and survival

Srikant Ambatipudi<sup>a,b,\*,1</sup>, Ryan Langdon<sup>a,b,1</sup>, Rebecca C. Richmond<sup>a,b</sup>, Matthew Suderman<sup>a,b</sup>, Devin C. Koestler<sup>c</sup>, Karl T. Kelsey<sup>d,e</sup>, Nabila Kazmi<sup>a,b</sup>, Christopher Penfold<sup>f</sup>, Karen M. Ho<sup>b</sup>, Wendy McArdle<sup>b</sup>, Susan M. Ring<sup>a,b</sup>, Miranda Pring<sup>f</sup>, Tim Waterboer<sup>g</sup>, Michael Pawlita<sup>g</sup>, Tom R. Gaunt<sup>a,b</sup>, George Davey Smith<sup>a,b</sup>, Steve Thomas<sup>f</sup>, Andy R. Ness<sup>f</sup>, Caroline L. Relton<sup>a,b</sup>

<sup>a</sup> MRC Integrative Epidemiology Unit, Bristol Medical School, University of Bristol, Bristol, UK

<sup>b</sup> Population Health Sciences, Bristol Medical School, University of Bristol, Bristol, UK

<sup>c</sup> Department of Biostatistics, University of Kansas Medical Center, Kansas City, KS, USA

<sup>d</sup> Department of Epidemiology, Brown University, School of Public Health, Providence, RI 02912, USA

<sup>e</sup> Department of Laboratory Medicine & Pathology, Brown University, Providence, RI 02912, USA

<sup>f</sup> National Institute for Health Research (NIHR) Bristol Biomedical Research Centre, University Hospitals Bristol NHS Foundation Trust and University of Bristol, Bristol, UK

<sup>g</sup> Division of Molecular Diagnostics of Oncogenic Infections, Infection, Inflammation and Cancer Program, German Cancer Research Center, Heidelberg, Germany

## ARTICLE INFO

### Keywords:

Head and neck cancer  
Systemic inflammation  
DNA methylation  
Neutrophil-to-lymphocyte ratio  
Lymphocyte-to-monocyte ratio  
Overall survival  
mdNLR

## ABSTRACT

**Objectives:** Head and neck squamous cell carcinoma (HNSCC) is often associated with chronic systemic inflammation (SI). In the present study, we assessed if DNA methylation-derived SI (mdSI) indices: Neutrophil-to-Lymphocyte ratio (mdNLR) and Lymphocyte-to-Monocyte ratio (mdLMR) are associated with the presence of HNSCC and overall survival (OS).

**Materials and methods:** We used two peripheral blood DNA methylation datasets: an HNSCC case-control dataset (n = 183) and an HNSCC survival dataset (n = 407) to estimate mdSI indices. We then performed multivariate regressions to test the association between mdSI indices, HNSCC development and OS.

**Results:** Multivariate logistic regression revealed that elevated mdNLR was associated with increased odds of being an HNSCC case (OR = 3.25, 95% CI = 2.14–5.34,  $P = 4 \times 10^{-7}$ ) while the converse was observed for mdLMR (OR = 0.88, 95% CI = 0.81–0.90,  $P = 2 \times 10^{-3}$ ).

In the HNSCC survival dataset, HPV16-E6 seropositive HNSCC cases had an elevated mdLMR ( $P = 9 \times 10^{-5}$ ) and a lower mdNLR ( $P = 0.003$ ) compared to seronegative patients. Multivariate Cox regression in the HNSCC survival dataset revealed that lower mdLMR (HR = 1.96, 95% CI = 1.30–2.95,  $P = 0.0013$ ) but not lower mdNLR (HR = 0.68, 95% CI = 0.46–1.00,  $P = 0.0501$ ) was associated with increased risk of death.

**Conclusion:** Our results indicate that mdSI estimated by DNA methylation data is associated with the presence of HNSCC and overall survival. The mdSI indices may be used as a valuable research tool to reliably estimate SI in the absence of cell-based estimates. Rigorous validation of our findings in large prospective studies is warranted in the future.

## Introduction

Head and neck squamous cell carcinoma (HNSCC) includes cancers arising from the lining epithelium of the upper aero-digestive tract including the oral cavity, larynx, pharynx, and nasopharynx [1]. Globally, HNSCC accounts for nearly 600,000 newly diagnosed cancer cases leading to approximately 325,000 deaths each year [2]. Tobacco and

alcohol abuse are the main risk factors for HNSCC [1]. However, high-risk types of human papillomavirus (HPV), especially HPV type 16 have emerged as risk factors in a subset of HNSCC, particularly oropharyngeal cancer (OPC) [3]. Disease stage has been the single most predictive prognostic factor for OPC [4], although recent studies point to HPV status as an independent marker of prognosis in HNSCC patients [5,6].

**Abbreviations:** mdNLR, Methylation derived Neutrophil-to-Lymphocyte Ratio; mdLMR, Methylation derived Lymphocyte-to-Monocyte Ratio

\* Corresponding author at: MRC Integrative Epidemiology Unit (IEU), Bristol Medical School, Oakfield House, Oakfield Grove, Bristol BS8 2BN, UK.

E-mail address: [s.ambatipudi@bristol.ac.uk](mailto:s.ambatipudi@bristol.ac.uk) (S. Ambatipudi).

<sup>1</sup> Authors contributed equally.

<https://doi.org/10.1016/j.oraloncology.2018.08.021>

Received 22 June 2018; Received in revised form 31 July 2018; Accepted 26 August 2018

Available online 05 September 2018

1368-8375/ © 2018 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Cancer related chronic systemic inflammation (SI) is an enabling characteristic of cancers which help them to acquire tumour hallmarks [7] and its role in cancer prognosis has been increasingly recognised [8]. Chronic SI also promotes tumour initiation and progression by induction of immunosuppression via Myeloid Derived Suppressor Cells (MDSCs) [9]. Indeed, HNSCC is often associated with immunosuppression, with an imbalance in both the composition and function of effector immune cells [10].

The SI response can be clinically measured by assessing circulating leukocyte ratios and/or acute phase proteins like C-reactive protein (CRP) [11,12]. In particular, the leukocyte subtype ratios like Neutrophil-to-Lymphocyte ratio (NLR) and Lymphocyte-to-Monocyte ratio (LMR) have shown promise as prognostic markers in many solid tumours [13] including HNSCC [14–22]. However, leukocyte measures are not readily available in many studies, especially in prospective cohorts [19].

DNA methylation based cell-type deconvolution algorithms have shown promise in estimating leukocyte cell type proportions [23]. Two recent studies have shown the utility of generating a methylation-derived NLR (mdNLR) index from peripheral blood DNA as a marker of cancer development and progression [19,24]. Furthermore, the authors reported a strong agreement between mdNLR and cell count based NLR estimates, instilling confidence in the DNA methylation based estimates of leukocytes and methylation-derived SI.

In the present study, we estimated mdSI indices (mdNLR and mdLMR) in pre-treatment HNSCC cases, cancer free controls, and in an independent set of HNSCC patients with overall survival (OS) data. We evaluated whether mdSI indices are associated with the presence of HNSCC and OS.

## Material and methods

### HNSCC case-control dataset

The DNA methylation data, percentage of leukocyte subtypes and covariates for the HNSCC case-control dataset were kindly provided by co-authors (DCK and KTK, GEO accession: GSE30229).

The study consisted of 92 pre-treatment HNSCC cases and 92 cancer-free control subjects with DNA methylation data from peripheral blood samples. The cases and controls were frequency-matched on age and gender. Details about the sample selection and preparation have been described previously [25]. DNA methylation was assessed using the Illumina Infinium HumanMethylation27 BeadChip assay (Illumina, Inc., CA, USA). To avoid potential biases, HNSCC case and control samples were randomized to bead chips. A sample with an unusually high value of mdLMR (mdLMR = 1116) was considered an outlier and was removed leaving a total of 91 HNSCC cases and 92 cancer-free controls for the final analysis.

### HNSCC survival dataset

The study population for this analysis was comprised of individuals enrolled in the Head and Neck 5000 clinical cohort study [26,27]. Briefly, 5511 people with a new head and neck cancer diagnosis were recruited from 76 centres across the UK between April 2011 and December 2014. Individuals were recruited before they started treatment unless their treatment was their diagnostic procedure. Full ethical approval was granted by The South West – Frenchay Regional Ethics Committee (ref: 10/H0107/57).

At baseline, participants were asked to complete three self-administered questions, which included questions on socio-economic circumstances, lifestyle, general health and past sexual behaviours. Biological samples (blood (n = 4676 (85%)), saliva (n = 4986 (90%)) and tissue) were collected from all consenting participants. Information on stage at diagnosis, treatment and various other clinical and pathological prognostic variables were abstracted from participants' medical

records. 5474 (99%) data capture forms and 4099 (74%) health and lifestyle questionnaires were completed [27].

The participants were selected for DNA methylation profiling based on ICD-10 coding of OPC (C01, C05.1, C05.2, C05.8, C09.0, C09.1, C09.8, C09.9, C10.0, C10.2, C10.3, C10.8, C10.9) and availability of OncoChip genotype data [28]. They were also selected on the basis of complete baseline questionnaire and data capture information, as well as the availability of both blood and saliva samples taken at baseline. To date, DNA samples isolated from buffy coats have been analysed for 448 participants.

Blood samples were frozen and stored at  $-80^{\circ}\text{C}$  and then processed in the Bristol Bioresource Laboratories. Following extraction, DNA was bisulphite-converted using the EZ DNA Methylation™ kit (Zymo, Irvine, CA, USA) as per the previously published protocol [29]. Following conversion, genome-wide methylation status of over 850,000 CpG sites was measured using the Infinium MethylationEPIC BeadChip [30]. The arrays were scanned using Illumina iScan and the initial quality review was assessed using GenomeStudio. DNA methylation data for the HNSCC survival dataset has not been previously published.

During the data generation process, a wide range of batch variables were recorded in a purpose-built laboratory information management system (LIMS).

### Quality control and normalisation

Raw data (IDAT files) from GenomeStudio were loaded into R package *meffil* [31] and quality control (QC) data extracted (Supplementary Fig. 1). In total, 5 samples failed at least one of the QC steps. Overall, 443 samples passed the QC. Due to the subsequent recoding of the ICD-10 classifications, we had 436 samples of the oral cavity and oropharyngeal cancers. After filtering for the samples with complete data on HPV status, alcohol consumption and smoking status, we were left with 407 samples for the final analysis. These samples consisted of 389 OPC and 18 oral cancer cases.

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.oraloncology.2018.08.021>.

Following QC, we performed functional normalization which exploits control probes to separate biological variation from technical variation [32]. Data were normalised using six control probe principal components derived from the technical probes.

### Tobacco, alcohol, comorbidity and HPV exposure

Detailed information on tobacco and alcohol history was obtained at baseline via the self-reported questionnaire. Participants were asked about their use of tobacco and alcohol products prior to receiving their HNSCC diagnosis.

Smoking status was defined as “ever” (current and former) or “never”. Former smokers were those that reported having smoked  $\geq 100$  cigarette in a lifetime, whilst never smokers were defined as having never smoked at least one daily cigarette during a whole year.

Respondents were asked to report their average weekly alcohol consumption and were defined as “ever” and “never”.

Chronic diseases are associated with increased systemic inflammation [33]. We used the Adult Comorbidity Evaluation 27 (ACE 27) completed by research nurses in clinical centres to record the presence and severity of medical comorbidities including chronic systemic diseases as described by Piccirillo et al. [34]. The participants were grouped into four categories: no co-morbidity, mild comorbidity; moderate decompensation and severe decompensation.

HPV serologic testing (HPV16 E6, E7, E1, E2, E4, and L1) was conducted at the German Cancer Research Center (DKFZ, Heidelberg, Germany) using glutathione S-transferase fusion protein-based multiplex serology [35]. HPV16 E6 antibodies have been shown to have potential diagnostic and/or prognostic capabilities in HPV-positive OPC [36]. We dichotomized the median fluorescence intensity (MFI) values to indicate HPV16 E6 seropositivity using a cut-off of  $\geq 1000$  MFI [37].

### Mortality data

Regular updates are received from the NHS Central Register (NHSCR) and the NHS Information Centre (NHSIC) notifying on subsequent cancer registrations and mortality among cohort members throughout the Head and Neck 5000 study. Recruitment for the study finished in December 2014 and follow-up information on mortality status was obtained in September 2017, resulting in at least 2.75 years of follow-up for all participants.

### Estimating cell counts and computing the methylation-derived systemic inflammation indices

For the HNSCC case-control dataset, cell counts were estimated as previously described [19,38]. For the HNSCC survival dataset, we used the dataset from Reinius et al. as a cell type reference [39] and cell counts were estimated using the Houseman et al. algorithm for estimating cell counts [38] in *meffil*. Each sample was normalised individually to the cell type reference, thus avoiding having cell count estimates dependent on other samples being included in the normalisation.

Methylation derived Neutrophil-to-Lymphocyte Ratio (mdNLR) was estimated by dividing estimated proportions of granulocytes by lymphocytes as previously described [19]. Similarly, methylation derived Lymphocyte-to-Monocyte Ratio (mdLMR) was estimated by dividing estimated proportions of lymphocytes by monocytes.

### Statistical analyses

The analyses were performed using statistical software R (version 3.4.0). A Wilcoxon rank sum test was used to compare the mean mdNLR and mdLMR in (a) HNSCC cases and cancer free controls (from the HNSCC case-control dataset) and (b) HNSCC cases with available OS data (from the HNSCC survival dataset).

Multivariate logistic regressions were performed to test the association between mdNLR (continuous), mdLMR (continuous) and HNSCC case-control status. To test the association of mdSI indices (categorical, above and below median) with OS, univariate and multivariate Cox proportional hazard analysis was performed in the HNSCC survival dataset.

Prior to testing associations in the HNSCC case-control dataset, any potential effect of plate and/or BeadChip were regressed out using ComBat [40] as previously described [19]. The multivariate logistic regression model was adjusted for covariates age, gender, smoking status and HPV status. The ability of mdSI indices to classify HNSCC cancer cases and cancer free controls was assessed using receiver operating characteristic (ROC) curves and corresponding Area Under the ROC Curve (AUC) values using the R package *pROC* [41].

For the HNSCC survival dataset, we performed univariate and multivariate Cox proportional hazard analyses using the R package *survival* (<https://cran.r-project.org/web/packages/survival/index.html>). For each model, the proportional hazards assumption for a Cox regression was tested to check for any violation using function *cox.zph* implemented in the R package.

The multivariate Cox proportional hazards regression model was adjusted for age, gender, smoking status (ever/never), tumour stage (stage I&II (low)/ III&IV (high)), HPV16 E6 serology (positive/negative), alcohol consumption (ever/never) and ACE27 categorisation. Furthermore, to address potential sources of unwanted technical variation, we performed Surrogate Variable Analysis (SVA) [42–44]. We created a full model matrix (a model matrix containing mdNLR (above/below median value), mdLMR (above/below median value), OS status, survival time, HPV16 E6 seropositivity status, smoking status, age, gender, alcohol consumption, ACE27 categorisation and low/high stage of tumour) and a null model matrix (a model matrix containing mdNLR (above/below median value), mdLMR (above/below median value), HPV16 E6 seropositivity status, smoking status, age, gender, alcohol

consumption, ACE27 categorisation and low/high stage of tumour) from our phenotype data. Ten surrogate variables were derived as the most variable technical artefacts in our data. The multivariate Cox proportional hazards regression model was further adjusted for these ten surrogate variables along with the covariates mentioned above. Kaplan-Meier survival curves were plotted using the R package *survminer* (<https://github.com/kassambara/survminer>).

Finally, a Wilcoxon rank sum test was performed to compare the mean values of myeloid differentiation associated 5 CpG sites [24] in HNSCC cases who died during the follow-up period and those who remained alive.

## Results

### Sample characteristics

#### HNSCC case-control dataset

The sample characteristics including demographic and clinical data for the HNSCC case-control dataset have been previously described [19] and are shown in Supplementary Table 1. The mean age of the participants was 60 years, with 69% men. The mean mdNLR and mdLMR were 2.35 (SD = 1.36) and 6.25 (SD = 5.37) respectively.

#### HNSCC survival dataset

The sample characteristics including demographic and clinical data for HNSCC are shown in Table 1. Four hundred and seven people with HNSCC were included in this study. The mean age of the participants was 59 years, with 77% men. For lifestyle associated risk factors, 97% were ever alcohol consumers, 69% had a smoking history (ever smokers). The majority of the tumours (85%) were of high stage (stage III or IV) vs low stage (stage I and II). Of all the HNSCC samples analysed for HPV16 E6 protein seropositivity, 67% of the samples were positive while 33% were negative. The mean mdNLR and mdLMR were 2.4 (SD = 2.51) and 3.5 (SD = 1.42), respectively.

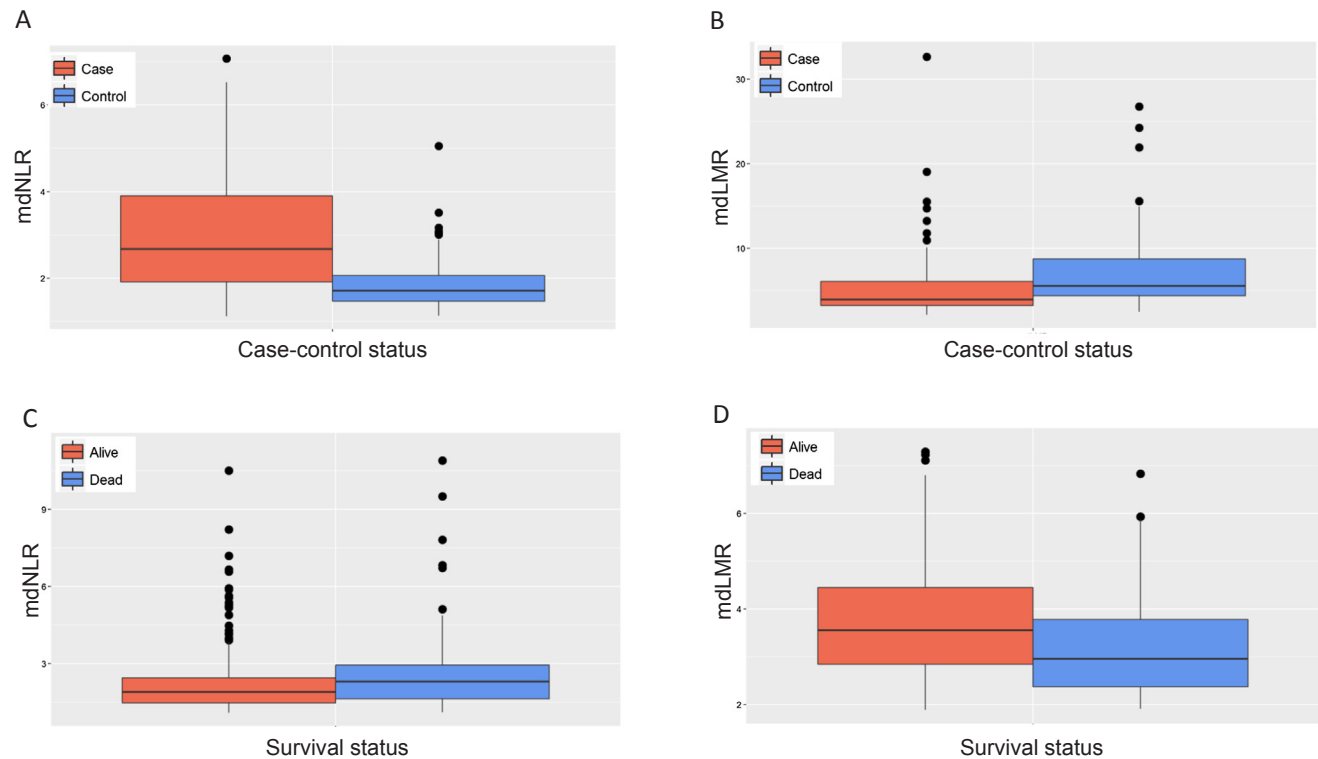
**Table 1**  
Sample characteristics of HNSCC survival dataset at baseline.

Characteristics		N (%)
Mean age (range)	59.24 (30–94)	407 (100%)
Mean mdNLR (SD)	2.43 (2.51)	
Mean mdLMR (SD)	3.45 (1.42)	
Gender	Male	319 (78%)
	Female	88 (22%)
Drinking status	Never-drinker	14 (3%)
	Ever-drinker	393 (97%)
Smoking status	Never-smoker	111 (27%)
	Ever-smoker	296 (73%)
Clinical stage	Low (TNM Stage 1 or 2)	55 (14%)
	High (TNM Stage 3 or 4)	352 (86%)
HPV16 E6 seropositivity	Absent	133 (33%)
	Present	274 (67%)
Tumour site	Oral cavity	18 (4%)
	Oropharynx	389 (96%)
Adult Comorbidity Evaluation 27 (ACE 27) categorisation	No comorbidity	212 (52%)
	Mild comorbidity	120 (30%)
	Moderate	65 (16%)
	decompensation	
	Severe decompensation	6 (1%)
	Unknown	4 (1%)

mdNLR = Methylation derived Neutrophil-to-Lymphocyte Ratio.

mdLMR = Methylation derived Lymphocyte-to-Monocyte Ratio.

SD = Standard Deviation.



**Fig. 1.** Methylation derived systemic inflammation indices in the HNSCC case-control and survival datasets. A. mdNLR in pre-treatment HNSCC cases and controls. B. mdLMR in pre-treatment HNSCC cases and controls. C. mdNLR in HNSCC cases who died during follow-up and those who were alive. D. mdLMR in HNSCC cases who died during follow-up and those who were alive.

*Methylation-derived systemic inflammation indices are associated with the risk of HNSCC*

We observed elevated estimated neutrophil ( $P = 1.57 \times 10^{-8}$ ) and monocyte ( $P = 0.0770$ ) counts and lower lymphocyte count ( $P = 6.49 \times 10^{-10}$ ) in HNSCC cases compared to controls (Supplementary Fig. 2A). A higher mdNLR was seen in HNSCC cases

compared to controls ( $P = 1.53 \times 10^{-9}$ , Fig. 1A), while elevated mdLMR was observed in controls compared to cases ( $P = 2.55 \times 10^{-7}$ , Fig. 1B). Using a multivariate logistic regression model including age, gender, smoking status and HPV16-E6 serology status as covariates, elevated mdNLR (OR = 3.25, 95% CI = 2.14–5.34,  $P = 4 \times 10^{-7}$ ) and a lower mdLMR (OR = 0.88, 95% CI = 0.81–0.90,  $P = 2.0 \times 10^{-3}$ )

**Table 2**  
Association between methylation-derived systemic inflammation indices (mdNLR, mdLMR) and case-control status in HNSCC dataset (n = 183).

Study variables	Univariate model OR (95% CI) (n = 183)	P-value	Multivariate model OR (95% CI) (n = 183)	P-value
<b>mdNLR (continuous)</b>	2.82 (1.96–4.33)	$2.22 \times 10^{-7}$	3.25 (2.14–5.34)	$4.03 \times 10^{-7}$
<b>Age</b>	–	–	0.98 (0.95–1.01)	0.12
<b>Gender</b>				
Female	–	–	Ref.	NA
Male	–	–	0.81 (0.37–1.77)	0.59
<b>Smoking status</b>	–	–		
Current	–	–	Ref.	NA
Former	–	–	1.04 (0.39–2.78)	0.94
Never	–	–	0.28 (0.08–0.88)	0.03
<b>HPV status</b>	–	–		
HPV16 E6 negative	–	–	Ref.	NA
HPV16 E6 positive	–	–	3.44 (1.41–8.92)	0.01
<b>mdLMR (continuous)</b>	0.91 (0.84–0.97)	0.014	0.88 (0.81–0.9)	$1.92 \times 10^{-3}$
<b>Age</b>	–	–	0.99 (0.96–1.0)	0.50
<b>Gender</b>				
Female	–	–	Ref.	NA
Male	–	–	0.52 (0.24–1.1)	0.09
<b>Smoking status</b>	–	–		
Current	–	–	Ref.	NA
Former	–	–	0.76 (0.30–1.9)	0.56
Never	–	–	0.23 (0.07–0.7)	0.01
<b>HPV status</b>	–	–		
HPV16 E6 negative	–	–	Ref.	NA
HPV16 E6 positive	–	–	4.80 (2.09–12.0)	$3.99 \times 10^{-4}$

mdNLR = Methylation derived Neutrophil-to-Lymphocyte Ratio.  
mdLMR = Methylation derived Lymphocyte-to-Monocyte Ratio.



were associated with increased odds of being a HNSCC case (Table 2). The methylation-derived systemic inflammation indices alone were able to distinguish HNSCC cases and controls compared to covariates: age, gender, smoking status and HPV status (Supplementary Fig. 3). Interestingly, mdNLR (AUC = 0.76 (95% CI = 0.69–0.83) and mdLMR (AUC = 0.72 (95% CI = 0.65–0.80) alone had similar AUC for distinguishing HNSCC cases from controls ( $P = 0.35$  for difference). Adding covariates to the systemic inflammation indices improved the mdNLR AUC in distinguishing HNSCC cases from controls compared to mdLMR (AUC<sub>mdNLR+cov</sub> = 0.82 (95% CI = 0.76–0.88), AUC<sub>mdLMR+cov</sub> = 0.75 (95% CI = 0.68–0.82),  $P = 0.05$  for difference) (Supplementary Fig. 3).

#### mdLMR is associated with overall survival in HNSCC

In the HNSCC survival dataset, 109 (27%) participants died during the median follow-up period of 4.54 years (range 0.18–6.41 years).

Leukocyte cell counts indicated that neutrophil ( $P = 0.02$ ) and monocyte ( $P = 9.9 \times 10^{-5}$ ) counts were elevated while lymphocyte count ( $P = 0.004$ ) was lower in HNSCC cases with poor survival (Supplementary Fig. 2B).

Elevated mdNLR was observed in HNSCC patients who died during the follow-up period ( $P = 0.004$ , Fig. 1C) while mdLMR was elevated in HNSCC patients who remained alive compared to those who died during follow-up ( $P = 1.1 \times 10^{-5}$ , Fig. 1D).

There were no serious violations of the proportionality assumption across the predictors used in the univariate and multivariate analysis. Univariate Cox proportional hazards regression analysis (Fig. 2 and Table 3) showed that lower mdNLR was associated with a reduced risk of death (HR = 0.55, 95% CI = 0.38–0.81,  $P = 0.00253$ ), while lower mdLMR was associated increased risk of death (HR = 2.38, 95% CI = 1.66–3.55,  $P = 0.00002$ ).

The results from our multivariate Cox proportional hazards regression analysis including age, gender, tumour stage, smoking status, HPV16 E6 serology status, alcohol consumption and ACE 27 categorisation as covariates, showed that lower mdNLR was not significantly associated with decreased risk of death (HR = 0.68, 95% CI = 0.46–1.00,  $P = 0.0501$ ) while the converse was observed for mdLMR (HR = 1.96, 95% CI = 1.30–2.95,  $P = 0.0013$ ), as shown in Table 3. Further adjustment for HNSCC tumour site (oral and OPC) did not attenuate our findings (Supplementary Table 2) for both mdNLR (HR = 0.69, 95% CI = 0.46–1.02,  $P = 0.0597$ ) and mdLMR (HR = 1.97, 95% CI = 1.31–2.97,  $P = 0.0012$ ). Importantly, adjusting for technical artefacts by surrogate variable analysis did not attenuate our findings for mdLMR (HR = 2.31, 95% CI = 1.41–3.77,  $P = 0.0009$ ), or mdNLR (HR = 0.65, 95% CI = 0.38–1.09,  $P = 0.1042$ ).

Chronological age, advanced stage (stage III/IV) and ever smoking (current/former) were associated with poorer OS in HNSCC. In

contrast, HPV16 E6 seropositivity (Table 3) and OPC (Supplementary Table 2) were associated with better OS. Elevated mdNLR and lower mdLMR were observed in HPV negative and ever smoker HNSCC cases (Supplementary Fig. 4).

We found that all five CpGs associated with myeloid cell differentiation (suggested to be a surrogate for mdNLR) were hypermethylated in HNSCC patients who remained alive during the follow-up period (Table 4, Supplementary Fig. 5).

## Discussion

In the present study, we have identified that methylation-derived systemic inflammation indices may be used to distinguish HNSCC cases and controls. The mdSI indices provide a slight improvement over covariates (age, gender, smoking and HPV status) in distinguishing HNSCC cases from controls. Intriguingly, lower mdLMR was associated with poorer OS.

We observed an elevated methylation-derived circulating neutrophil and monocyte count and a decreased lymphocyte count in HNSCC cases compared to controls. Similarly, HNSCC cases with poor OS showed elevated neutrophil and monocyte cell counts and a lower lymphocyte count. Our findings concur with previous reports on cell count-based leukocyte measurements in HNSCC development and progression [45–47].

We utilised mdNLR and mdLMR to understand the contribution of systemic inflammation in HNSCC development and survival. Findings from the present study suggests an association of mdSI indices with the presence of HNSCC and OS similar to previous cell count based reports [16,18]. Interestingly, our DNA methylation-derived estimates of NLR and LMR were similar to the cell count based measure of SI [18,48]. The similarities between DNA methylation and cell count based inflammation indices strengthens the utility of mdSI indices as a valuable research tool to estimate SI in the absence of cell count based measurement, especially in prospective studies.

Although the mdSI indices are associated with the presence of HNSCC and with OS, these associations may also be driven by exposure to inflammation-associated risk factors of HNSCC such as smoking and HPV status that are also associated with poor prognosis in HNSCC [6]. Our findings of an elevated mdNLR and lower mdLMR in HPV negative and ever smoking HNSCCs are in agreement with the previous observations [14]. These observations may be indicative of the potential biological differences between HPV positive and negative tumours. HPV effectively evades the innate immune system by confining gene and protein synthesis to the epithelial cells hence, only nominal amounts of replicating virus are exposed to the immune system [49,50]. Our findings of elevated mdLMR and lower mdNLR in HPV-positive HNSCC may therefore be reflective of an innate immune response.

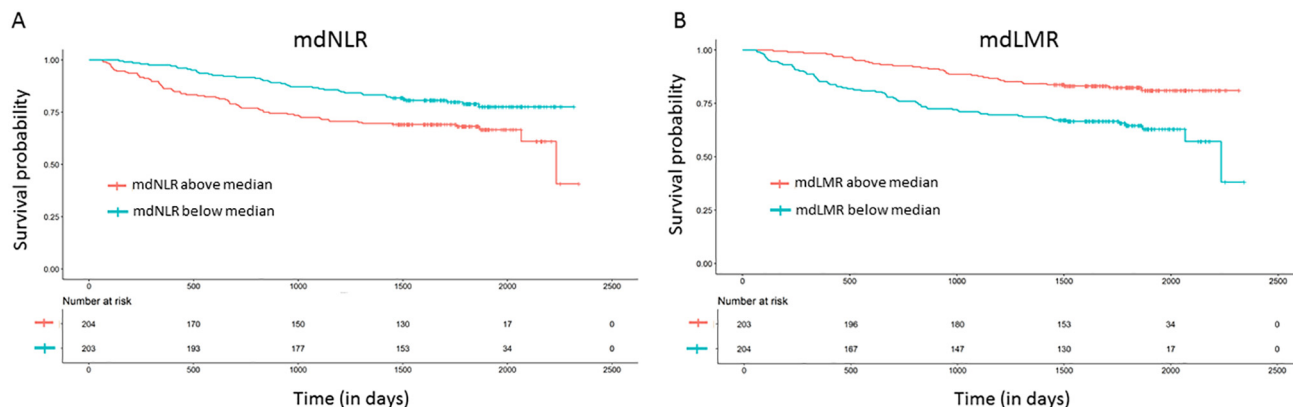


Fig. 2. Overall survival curve for methylation derived systemic inflammation indices A. mdNLR (above and below median) and B. mdLMR (above and below median).

**Table 3**

Association between methylation-derived systemic inflammation indices (mdNLR, mdLMR) and overall survival in HNSCC cases.

Study variables	Overall survival (n = 407)			
	Univariate model HR (95% CI)	P-value	Multivariate model HR (95% CI)	P-value
<b>mdNLR (below median vs. above median)</b>	0.55 (0.38–0.81)	0.0025	0.68 (0.46–1.00)	0.0501
<b>Age</b>	–	–	1.03 (1.01–1.05)	0.0009
<b>Gender</b>				
Female	–	–	Ref.	NA
Male	–	–	1.07 (0.66–1.73)	0.7986
<b>Stage</b>				
Low (T1&T2)	–	–	Ref.	NA
High (T3&T4)	–	–	2.62 (1.34–5.12)	0.0050
<b>Smoking status</b>				
Never	–	–	Ref.	NA
Ever	–	–	2.21 (1.20–4.09)	0.0110
<b>HPV status</b>				
HPV16 E6 negative	–	–	Ref.	NA
HPV16 E6 positive	–	–	0.32 (0.22–0.49)	$4.9 \times 10^{-8}$
<b>Drinking</b>				
Never	–	–	Ref.	NA
Ever	–	–	1.03 (0.67–1.58)	0.9024
<b>Adult Comorbidity Evaluation 27 (ACE 27) categorisation</b>				
No co-morbidity	–	–	Ref.	NA
Mild comorbidity	–	–	1.49 (0.92–2.41)	0.1014
Moderate decompensation	–	–	1.50 (0.88–2.55)	0.1323
Severe decompensation	–	–	4.66 (1.75–12.43)	0.0021
Unknown	–	–	5.23 (1.21–22.63)	0.0270
<b>mdLMR (below median vs. above median)</b>	2.38 (1.59–3.55)	$2.1 \times 10^{-5}$	1.96 (1.30–2.95)	0.0013
<b>Age</b>	–	–	1.03 (1.01–1.05)	0.0141
<b>Gender</b>				
Female	–	–	Ref.	NA
Male	–	–	1.16 (0.71–1.89)	0.5610
<b>Stage</b>				
Low (T1&T2)	–	–	Ref.	NA
High (T3&T4)	–	–	2.56 (1.30–5.02)	0.0063
<b>Smoking status</b>				
Never	–	–	Ref.	NA
Ever	–	–	2.34 (1.26–4.34)	0.0069
<b>HPV status</b>				
HPV16 E6 negative	–	–	Ref.	NA
HPV16 E6 positive	–	–	0.33 (0.22–0.49)	$5.5 \times 10^{-8}$
<b>Drinking</b>				
Never	–	–	Ref.	NA
Ever	–	–	1.11 (0.72–1.73)	0.6343
<b>Adult Comorbidity Evaluation 27 (ACE 27) categorisation</b>				
No co-morbidity	–	–	Ref.	NA
Mild comorbidity	–	–	1.44 (0.89–2.32)	0.1365
Moderate decompensation	–	–	1.44 (0.84–2.45)	0.1854
Severe decompensation	–	–	4.53 (1.71–12.05)	0.0024
Unknown	–	–	5.00 (1.14–21.94)	0.0327

mdNLR = Methylation derived Neutrophil-to-Lymphocyte Ratio.

mdLMR = Methylation derived Lymphocyte-to-Monocyte Ratio.

**Table 4**

DNA methylation (beta values) of myeloid cell differentiation associated CpGs in HNSCC survival dataset.

Myeloid differentiation CpGs	Mean Alive (SD)	Mean Dead (SD)	P-value <sup>a</sup>
cg00901982	0.23 (0.09)	0.20 (0.09)	0.0007
cg25938803	0.28 (0.08)	0.26 (0.08)	0.0012
cg01591037	0.31 (0.10)	0.29 (0.10)	0.0249
cg03621504	0.20 (0.08)	0.18 (0.08)	0.0313
cg10456459	0.33 (0.11)	0.28 (0.10)	0.0003

<sup>a</sup> Wilcoxon rank sum test.

Smoking is associated with increased systemic inflammation [51]. Lifetime smoking related tobacco exposure measured by pack years was recently shown to be associated with elevated NLR [52]. Indeed, our recent DNA methylation study identified an altered number of immune cells in response to smoking [53].

We observed an association between mdSI indices (elevated mdNLR

and lower mdLMR) and increased odds of being an HNSCC case. Our results concur with the previously published work validating the SI indices derived using DNA methylation data [14–16,18]. Importantly, the level of mdNLR derived inflammation index were similar to the NLR derived using cell counts in HNSCC [16,48]. Previous studies have reported a higher monocyte count and a lower lymphocyte count associated with poor clinical outcome in HNSCC [54–56]. Our finding of lower LMR associated with reduced OS validates previous reports that cell count based pre-treatment LMR may be an independent prognostic marker in cancers, including HNSCC [18,57].

Altered SI (NLR, LMR) derived from either DNA methylation-based data or based on cell counts are reflective of systemic inflammation [13]. Previous studies have suggested a vicious cycle of interaction between tumour cells and cells of myeloid origin such as neutrophils and monocytes through cytokines which leads to neo-angiogenesis and poor treatment response [46,47,58,59]. On the other hand, lymphocytes play a critical role in strengthening the host immune response against cancer [55]. In fact, the levels of tumour infiltrating

lymphocytes (TILs) are known to predict survival in OPC patients [60]. We observed an increase in baseline methylation-derived myeloid cell counts (neutrophils and monocytes) and a decrease in baseline lymphocytes in incident HNSCC cases and cases with poor survival. Thus, our findings underline the significance of immune homeostasis in HNSCC development and progression.

Strengths of our study include the use of two datasets, giving us the ability to explore varied roles for mdSI indices in distinguishing pre-treatment HNSCC cases and controls, as well as in relation to survival. We performed multivariate analyses adjusting for appropriate potential confounders and possible sources of technical variation.

Our study is not without limitations. Firstly, the sample size for both the case-control and OS studies was small, moreover, we were unable to identify independent prospective datasets to validate our findings. This is attributed to limited published and publicly-available HNSCC datasets with genome-wide DNA methylation information on whole blood. Secondly, in the absence of genetic instruments for the cell count based systemic inflammation index, we are unable to evaluate causality of the observed association using Mendelian randomization [61] nor can we rule out reverse causation in the HNSCC case-control study. Thirdly, we were unable to compare mdSI indices to cell count based SI indices due to the lack of availability of directly measured blood cell type proportions for the studied datasets. In spite of these limitations, the confidence in our measured methylation-derived SI is strengthened by (i) previous studies that have validated the use methylation-derived cell counts in estimating SI [19,24] and (ii) similarities between our mdSI indices and previously published cell count based SI. Finally, we had limited information on the presence of oral inflammatory conditions such as oral lichen planus, Behcet's disease, and recurrent aphthous stomatitis in our datasets, so we were unable to adjust for these factors in the statistical models. However, we did account for the presence of chronic diseases associated with inflammatory conditions by adjusting for the ACE-27 score in our statistical models.

In conclusion, we have demonstrated that systemic inflammation indices are associated with the presence of HNSCC. Further, the mdSI indices are sufficient to distinguish HNSCC case and controls. In the HNSCC survival dataset, lower mdLMR was associated with poorer OS. The mdSI indices may be useful as a research tool for predicting high-risk HNSCC, especially HPV-negative HNSCC where there is a lack of reliable biomarkers of detection, although this would require rigorous validation in large prospective studies. The mdSI indices will be particularly helpful in prospective studies where the estimates of leukocyte subtypes were not recorded at recruitment. It remains to be tested whether mdSI measures SI independent of acute phase proteins such as CRP. Finally, we would be interested in testing whether mdSI in circulation is reflective of inflammation status in tumours.

## Conflict of interest

The authors declare that they have no conflict of interest.

## Acknowledgements

We would like to thank the people with head and neck cancer who took part in this study. We would also like to thank the research, laboratory and clinical staff who supported this study.

## Funding

This publication presents data from the Head and Neck 5000 study. The study was a component of independent research funded by the National Institute for Health Research (NIHR) under its Programme Grants for Applied Research scheme [RP-PG-0707-10034]. Human papillomavirus (HPV) serology was supported by a Cancer Research UK Programme Grant, the Integrative Cancer Epidemiology Programme [grant number C18281/A19169]. SA's work is supported by Cancer

Research UK [grant number C18281/A19169]. SA, RL, RCR, MS, NK, CP, KH, WM, SMR, TG, GDS and CLR work in the Medical Research Council Integrative Epidemiology Unit at the University of Bristol which is supported by the Medical Research Council and the University of Bristol [grant number MC\_UU\_00011/1, MC\_UU\_00011/4 and MC\_UU\_00011/5]. The study is also supported by the US National Institutes of Health [grant number CA078609].

## Role of the funding source

The views expressed in this publication are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. The funders had no role in study design, data collection, analysis, and preparation of the manuscript.

## References

- [1] Leemans CR, Braakhuis BJ, Brakenhoff RH. The molecular biology of head and neck cancer. *Nat Rev Cancer* 2011;11:9–22.
- [2] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, et al. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* 2015;136:E359–86.
- [3] Gillison ML, Koch WM, Capone RB, Spafford M, Westra WH, Wu L, et al. Evidence for a causal association between human papillomavirus and a subset of head and neck cancers. *J Natl Cancer Inst* 2000;92:709–20.
- [4] Woolgar JA. Histopathological prognosticators in oral and oropharyngeal squamous cell carcinoma. *Oral Oncol* 2006;42:229–39.
- [5] Rischin D, Ferris RL, Le QT. Overview of advances in head and neck cancer. *J Clin Oncol* 2015;33:3225–6.
- [6] Ang KK, Harris J, Wheeler R, Weber R, Rosenthal DI, Nguyen-Tan PF, et al. Human papillomavirus and survival of patients with oropharyngeal cancer. *N Engl J Med* 2010;363:24–35.
- [7] Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell* 2011;144:646–74.
- [8] Roxburgh CS, McMillan DC. Role of systemic inflammatory response in predicting survival in patients with primary operable cancer. *Future Oncol* 2010;6:149–63.
- [9] Wang D, DuBois RN. Immunosuppression associated with chronic inflammation in the tumor microenvironment. *Carcinogenesis* 2015;36:1085–93.
- [10] Hanna GJ, Adkins DR, Zolkind P, Uppaluri R. Rationale for neoadjuvant immunotherapy in head and neck squamous cell carcinoma. *Oral Oncol* 2017;73:65–9.
- [11] Gabay C, Kushner I. Acute-phase proteins and other systemic responses to inflammation. *N Engl J Med* 1999;340:448–54.
- [12] Roxburgh CS, McMillan DC. Cancer and systemic inflammation: treat the tumour and treat the host. *Br J Cancer* 2014;110:1409–12.
- [13] Templeton AJ, McNamara MG, Seruga B, Vera-Badillo FE, Aneja P, Ocana A, et al. Prognostic role of neutrophil-to-lymphocyte ratio in solid tumors: a systematic review and meta-analysis. *J Natl Cancer Inst* 2014;106:dju124.
- [14] Rachidi S, Wallace K, Wrangle JM, Day TA, Alberg AJ, Li Z. Neutrophil-to-lymphocyte ratio and overall survival in all sites of head and neck squamous cell carcinoma. *Head Neck* 2016;38(Suppl 1):E1068–74.
- [15] Rassouli A, Saliba J, Castano R, Hier M, Zeitouni AG. Systemic inflammatory markers as independent prognosticators of head and neck squamous cell carcinoma. *Head Neck* 2015;37:103–10.
- [16] Rosculet N, Zhou XC, Ha P, Tang M, Levine MA, Neuner G, et al. Neutrophil-to-lymphocyte ratio: Prognostic indicator for head and neck squamous cell carcinoma. *Head Neck* 2017;39:662–7.
- [17] Charles KA, Harris BD, Haddad CR, Clarke SJ, Guminski A, Stevens M, et al. Systemic inflammation is an independent predictive marker of clinical outcomes in mucosal squamous cell carcinoma of the head and neck in oropharyngeal and non-oropharyngeal patients. *BMC Cancer* 2016;16:124.
- [18] Kano S, Homma A, Hatakeyama H, Mizumachi T, Sakashita T, Kakizaki T, et al. Pretreatment lymphocyte-to-monocyte ratio as an independent prognostic factor for head and neck cancer. *Head Neck* 2017;39:247–53.
- [19] Koestler DC, Usset J, Christensen BC, Marsit CJ, Karagas MR, Kelsey KT, et al. DNA methylation-derived neutrophil-to-lymphocyte ratio: an epigenetic tool to explore cancer inflammation and outcomes. *Cancer Epidemiol Biomarkers Prev* 2017;26:328–38.
- [20] Perisanidis C, Kornel G, Poschl PW, Holzinger D, Pirklbauer K, Schopper C, et al. High neutrophil-to-lymphocyte ratio is an independent marker of poor disease-specific survival in patients with oral cancer. *Med Oncol* 2013;30:334.
- [21] Proctor MJ, Morrison DS, Talwar D, Balmer SM, Fletcher CD, O'Reilly DS, et al. A comparison of inflammation-based prognostic scores in patients with cancer. A Glasgow Inflammation Outcome Study. *Eur J Cancer* 2011;47:2633–41.
- [22] Takenaka Y, Oya R, Kitamiura T, Ashida N, Shimizu K, Takemura K, et al. Prognostic role of neutrophil-to-lymphocyte ratio in head and neck cancer: A meta-analysis. *Head Neck* 2017.
- [23] Titus AJ, Gallimore RM, Salas LA, Christensen BC. Cell-type deconvolution from DNA methylation: a review of recent applications. *Hum Mol Genet* 2017;26:R216–24.
- [24] Wiencke JK, Koestler DC, Salas LA, Wiemels JL, Roy RP, Hansen HM, et al.



- Immunomethylomic approach to explore the blood neutrophil lymphocyte ratio (NLR) in glioma survival. *Clin Epigenetics* 2017;9:10.
- [25] Langevin SM, Koestler DC, Christensen BC, Butler RA, Wiencke JK, Nelson HH, et al. Peripheral blood DNA methylation profiles are indicative of head and neck squamous cell carcinoma: an epigenome-wide association study. *Epigenetics* 2012;7:291–9.
- [26] Ness AR, Waylen A, Hurley K, Jeffreys M, Penfold C, Pring M, et al. Establishing a large prospective clinical cohort in people with head and neck cancer as a biomedical resource: head and neck 5000. *BMC Cancer* 2014;14:973.
- [27] Ness AR, Waylen A, Hurley K, Jeffreys M, Penfold C, Pring M, et al. Recruitment, response rates and characteristics of 5511 people enrolled in a prospective clinical cohort study: head and neck 5000. *Clin Otolaryngol* 2016;41:804–9.
- [28] Lesueur C, Diergaarde B, Olshan AF, Wunsch-Filho V, Ness AR, Liu G, et al. Genome-wide association analyses identify new susceptibility loci for oral cavity and pharyngeal cancer. *Nat Genet* 2016;48:1544–50.
- [29] Relton CL, Gaunt T, McArdle W, Ho K, Duggirala A, Shihab H, et al. Data Resource Profile: Accessible Resource for Integrated Epigenomic Studies (ARIES). *Int J Epidemiol* 2015;44:1181–90.
- [30] Pidsley R, Zotenko E, Peters TJ, Lawrence MG, Risbridger GP, Molloy P, et al. Critical evaluation of the Illumina MethylationEPIC BeadChip microarray for whole-genome DNA methylation profiling. *Genome Biol* 2016;17:208.
- [31] Min Josine, Hemani G, Smith George Davey, Relton Caroline L, Suderman Matthew. Meffil: efficient normalisation and analysis of very large DNA methylation samples. *bioRxiv* 2017:125963.
- [32] Fortin JP, Labbe A, Lemire M, Zanke BW, Hudson TJ, Fertig EJ, et al. Functional normalization of 450k methylation array data improves replication in large cancer studies. *Genome Biol* 2014;15:503.
- [33] Ambatipudi S, Sharp GC, Clarke SLN, Plant D, Tobias JH, Evans DM, et al. Assessing the Role of DNA Methylation-Derived Neutrophil-to-Lymphocyte Ratio in Rheumatoid Arthritis. *J Immunol Res* 2018;2018:10. <https://doi.org/10.1155/2018/2624981>.
- [34] Piccirillo JF, Tierney RM, Costas I, Grove L, Spitznagel Jr. EL. Prognostic importance of comorbidity in a hospital-based cancer registry. *JAMA* 2004;291:2441–7.
- [35] Waterboer T, Sehr P, Michael KM, Franceschi S, Nieland JD, Joos TO, et al. Multiplex human papillomavirus serology based on in situ-purified glutathione s-transferase fusion proteins. *Clin Chem* 2005;51:1845–53.
- [36] Lang Kuhs KA, Kreimer AR, Trivedi S, Holzinger D, Pawlita M, Pfeiffer RM, et al. Human papillomavirus 16 E6 antibodies are sensitive for human papillomavirus-driven oropharyngeal cancer and are associated with recurrence. *Cancer* 2017;123:4382–90.
- [37] Kreimer AR, Johansson M, Waterboer T, Kaaks R, Chang-Claude J, Drogen D, et al. Evaluation of human papillomavirus antibodies and risk of subsequent head and neck cancer. *J Clin Oncol* 2013;31:2708–15.
- [38] Houseman EA, Accomando WP, Koestler DC, Christensen BC, Marsit CJ, Nelson HH, et al. DNA methylation arrays as surrogate measures of cell mixture distribution. *BMC Bioinf* 2012;13:86.
- [39] Reinis LE, Acevedo N, Joerink M, Pershagen G, Dahlen SE, Greco D, et al. Differential DNA methylation in purified human blood cells: implications for cell lineage and studies on disease susceptibility. *PLoS ONE* 2012;7:e41361.
- [40] Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. *Biostatistics* 2007;8:118–27.
- [41] Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinf* 2011;12:77.
- [42] Leek JT, Johnson WE, Parker HS, Jaffe AE, Storey JD. The sva package for removing batch effects and other unwanted variation in high-throughput experiments. *Bioinformatics* 2012;28:882–3.
- [43] McGregor K, Bernatsky S, Colmegna I, Hudson M, Pastinen T, Labbe A, et al. An evaluation of methods correcting for cell-type heterogeneity in DNA methylation studies. *Genome Biol* 2016;17:84.
- [44] Perrier F, Novoloaca A, Ambatipudi S, Baglietto L, Ghantous A, Perduca V, et al. Identifying and correcting epigenetics measurements for systematic sources of variation. *Clin Epigenetics* 2018;10:38.
- [45] Trellakis S, Bruderek K, Dumitru CA, Gholaman H, Gu X, Bankfalvi A, et al. Polymorphonuclear granulocytes in human head and neck cancer: enhanced inflammatory activity, modulation by cancer cells and expansion in advanced disease. *Int J Cancer* 2011;129:2183–93.
- [46] Dumitru CA, Lang S, Brandau S. Modulation of neutrophil granulocytes in the tumor microenvironment: mechanisms and consequences for tumor progression. *Semin Cancer Biol* 2013;23:141–8.
- [47] Huang SH, Waldron JN, Milosevic M, Shen X, Ringash J, Su J, et al. Prognostic value of pretreatment circulating neutrophils, monocytes, and lymphocytes in oropharyngeal cancer stratified by human papillomavirus status. *Cancer* 2015;121:545–55.
- [48] Nakashima H, Matsuoka Y, Yoshida R, Nagata M, Hirose A, Kawahara K, et al. Pretreatment neutrophil to lymphocyte ratio predicts the chemoradiotherapy outcome and survival in patients with oral squamous cell carcinoma: a retrospective study. *BMC Cancer* 2016;16:41.
- [49] Stanley MA. Immune responses to human papilloma viruses. *Indian J Med Res* 2009;130:266–76.
- [50] Kanodia S, Fahey LM, Kast WM. Mechanisms used by human papillomaviruses to escape the host immune response. *Curr Cancer Drug Targets* 2007;7:79–89.
- [51] Bakhr A, Erlinger TP. Smoking cessation and cardiovascular disease risk factors: results from the Third National Health and Nutrition Examination Survey. *PLoS Med* 2005;2:e160.
- [52] Tulgar YK, Cakar S, Tulgar S, Dalkilic O, Cakiroglu B, Uyanik BS. The effect of smoking on neutrophil/lymphocyte and platelet/lymphocyte ratio and platelet indices: a retrospective study. *Eur Rev Med Pharmacol Sci* 2016;20:3112–8.
- [53] Ambatipudi S, Cuenin C, Hernandez-Vargas H, Ghantous A, Le Calvez-Kelm F, Kaaks R, et al. Tobacco smoking-associated genome-wide DNA methylation changes in the EPIC study. *Epigenomics* 2016;8:599–618.
- [54] Tsai YD, Wang CP, Chen CY, Lin LW, Hwang TZ, Lu LF, et al. Pretreatment circulating monocyte count associated with poor prognosis in patients with oral cavity cancer. *Head Neck* 2014;36:947–53.
- [55] Wallis SP, Stafford ND, Greenman J. Clinical relevance of immune parameters in the tumor microenvironment of head and neck cancers. *Head Neck* 2015;37:449–59.
- [56] Dewyer NA, Wolf GT, Light E, Worden F, Urba S, Eisbruch A, et al. Circulating CD4-positive lymphocyte levels as predictor of response to induction chemotherapy in patients with advanced laryngeal cancer. *Head Neck* 2014;36:9–14.
- [57] Gu L, Li H, Chen L, Ma X, Li X, Gao Y, et al. Prognostic role of lymphocyte to monocyte ratio for patients with cancer: evidence from a systematic review and meta-analysis. *Oncotarget* 2016;7:31926–42.
- [58] Ahn GO, Brown JM. Matrix metalloproteinase-9 is required for tumor vasculogenesis but not for angiogenesis: role of bone marrow-derived myelomonocytic cells. *Cancer Cell* 2008;13:193–205.
- [59] Dirix AE, Oude Egbrink MG, Wagstaff J, Griffioen AW. Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. *J Leukoc Biol* 2006;80:1183–96.
- [60] Ward MJ, Thirdborough SM, Mellows T, Riley C, Harris S, Suchak K, et al. Tumour-infiltrating lymphocytes predict for outcome in HPV-positive oropharyngeal cancer. *Br J Cancer* 2014;110:489–500.
- [61] Davey Smith G, Hemani G. Mendelian randomization: genetic anchors for causal inference in epidemiological studies. *Hum Mol Genet* 2014;23:R89–98.